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RESEARCH ARTICLE

Pregnancy lipidomic profiles and DNA methylation in newborns from the CHAMACOS cohort

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Abstract

Lipids play a role in many biological functions and the newly emerging field of lipidomics aims to characterize the varying classes of lipid molecules present in biological specimens. Animal models have shown associations between maternal dietary supplementation with fatty acids during pregnancy and epigenetic changes in their offspring, demonstrating a mechanism through which prenatal environment can affect outcomes in children; however, data on maternal lipid metabolite levels during pregnancy and newborn DNA methylation in humans are sparse. In this study, we assessed the relationship of maternal lipid metabolites measured in the blood from pregnant women with newborn DNA methylation profiles in the Center for the Health Assessment of Mothers and Children of Salinas cohort. Targeted metabolomics was performed by selected reaction monitoring liquid chromatography and triple quadrupole mass spectrometry to measure 92 metabolites in plasma samples of pregnant women at ~26 weeks gestation. DNA methylation was assessed using the Infinium HumanMethylation 450K BeadChip adjusting for cord blood cell composition. We uncovered numerous false discovery rate significant associations between maternal metabolite levels, particularly phospholipid and lysolipid metabolites, and newborn methylation. The majority of the observed relationships were negative, suggesting that higher lipid metabolites during pregnancy are associated with lower methylation levels at genes related to fetal development. These results further elucidate the complex relationship between early life exposures, maternal lipid metabolites, and infant epigenetic status.

Key words: DNA methylation; metabolomics; Mexican-American; epigenetics; newborns; cord blood; prenatal exposure

Introduction

Metabolomics has emerged as a useful tool to examine the profiles of small molecule metabolites in biospecimens [1]. Factors that have been associated with metabolite levels include age [2], diseases such as obesity [3], genetic variants throughout the human genome [4], and epigenetics [5, 6]. Both untargeted and targeted methodologies are commonly used in metabolomic research, with the former measuring thousands of metabolites in a biological sample and the latter focusing on a subset of metabolites, often involved in specific biological pathways [2]. For instance, lipidomics evaluates the function and distribution of lipids, which have a greater diversity of molecular species

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compared to the other classes of biological molecules in the body, such as carbohydrates and amino acids [7].

Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and non-coding RNAs [8]. DNA methylation is the most commonly examined epigenetic mechanism and refers to the addition of a methyl group to the C5 position of the cytosine ring in a CpG dinucleotide by DNA methyltransferases [9]. DNA methylation patterns are retained following somatic cell division [10]. In the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort and as part of the Pregnancy and Childhood Epigenetics consortium, we have explored the relationship of infant DNA methylation with early life environmental exposure of mothers to chemicals during pregnancy, including endocrine disrupting chemicals [11–13] and smoking [14]. Since the epigenome undergoes remodeling and rapid cell division in the prenatal period [8], this life stage is considered particularly sensitive to environmental insults and can have implications for disease trajectories [15].

There remains a paucity of information on the relationship between metabolite profiles, in general and specific to the lipid species, and DNA methylation, particularly the link between maternal lipid metabolites during pregnancy and offspring epigenetics, which could be a mechanism through which the *in utero* environment exerts an effect on epigenetic mechanisms that could impact offspring health. Most of the existing research on this topic has been conducted in animal models and has focused on the relationship between maternal nutrition, including dietary supplementation with fatty acids, and offspring methylation status [16, 17]. Only one study to date has examined the association between *in utero* metabolic traits, including lipid metabolites, and DNA methylation profiles in newborns [5]. The authors found significant relationships between maternal fatty acid levels and newborn methylation globally and at target genes related to early growth.

Previous research in the CHAMACOS cohort examined the association between maternal prenatal phthalate urine metabolite levels and targeted metabolomic compounds present in plasma and urine, collected at 26 weeks gestation [18]. In addition, we assessed the relationship between pre-pregnancy body mass index (BMI) and maternal metabolomics profiles. We observed numerous associations of metabolomic markers involved in lipid and nucleic acid metabolism and the inflammatory response with both urinary phthalate metabolites and maternal BMI. This study aims to determine the association between maternal metabolomic profiles in pregnancy and child DNA methylation at birth, emphasizing metabolites enriched for lipid pathways that were confirmed as relevant in previous work in CHAMACOS [18]. This study will contribute to the growing body of evidence of the influence of the early life environment on epigenetic mechanisms.

Results

Study Population

Characteristics of CHAMACOS mothers and children included in the study are presented in Table 1. Additional data on CHAMACOS demographic parameters have been previously described in [19]. Most mothers were overweight or obese (BMI $\geq$25 kg/m^2) prior to pregnancy. Roughly equal portions of children were boys and girls, 5% were of low birth weight (<2500 g) and 7% born were premature (<37 weeks).

Table 1: characteristics of CHAMACOS children and mothers ($N = 81$ pairs) with newborn 450K data and assessment of maternal metabolomic profiles at 26 weeks gestation

Characteristics	
	N (%)
Maternal age at delivery	
18–24	32 (39.5)
25–29	36 (44.4)
30–34	11 (13.6)
35–45	2 (2.5)
Newborn sex	
Boy	39 (48.1)
Girl	42 (51.9)
	Mean (SD)
Maternal pre-pregnancy BMI (kg/m^2)	25.5 (4.3)
Newborn gestational age (weeks)	38.8 (1.7)
Newborn birth weight (grams)	3413.1 (558.0)
Newborn white blood cell count (%)	
Granulocytes	46.4 (10.3)
CD4+ T	15.5 (6.7)
CD8+ T	13.2 (3.8)
B cells	9.5 (3.2)
nRBC	10.4 (6.5)
Monocytes	8.1 (1.9)
NK cells	0.4 (1.2)

Maternal Prenatal Metabolomic Profiles

In this study, we focused on maternal metabolites previously identified to be relevant to lipid biosynthesis, arachidonate enrichment and release, and inflammatory signaling [18]. The 92 plasma metabolites in the study included fatty acids, lysolipids, phospholipids, sphingolipids, monoacylglycerols, diacylglycerols, and triacylglycerols (Table 2, Figs 1 and 2). Example distributions of specific fatty acid, lysolipid, and phospholipid metabolites are shown in Fig. 1. Median lipid metabolite counts ranged from 0.01 (C16:0e/C18:1 PSe) to 44 132.92 (C16:0/C18:1 PC) (Table 2). In Fig. 2, we plotted the median values for the metabolites within each of the four quartiles based on the ranges in the metabolite distributions. The phosphatidylcholine (PC) phospholipids had the highest median values and a broad range across subjects. All metabolites were log(1+x) transformed in analyses to reduce the influence of outliers.

Maternal Metabolomics and Newborn DNA Methylation Analyses

Numerous maternal prenatal metabolites were significantly associated, after correcting for multiple hypotheses testing, with DNA methylation of CHAMACOS newborns (Table 3). The metabolites that were related to newborn DNA methylation after adjusting for relevant covariates, indicated by an asterisk above the bar plots in Fig. 2, were distributed across the four quartiles based on metabolite ranges, with four of the nine significant metabolites found in the lowest quartile of ranges (Fig. 2A). After adjusting for child sex, batch, and white blood cell type estimates, four phospholipids (C16:0/C20:4 PS, C16:0e/C18:1 PSe, C18:0/C20:4 alkyl PA, C18:0e/C18:1 PEe), four lysolipids (C16:0 alkyl LPA, C18:1 alkyl LPA, C18:1 LPI, C20:4 LPS), and the fatty acid C18:0 FFA were among the significant metabolites related to newborn methylation. The number of CpG sites significantly associated with a specific metabolite ranged from 1 to 6. The phospholipid C18:0/C20:4 alkyl PA, shown in Fig. 3, was one

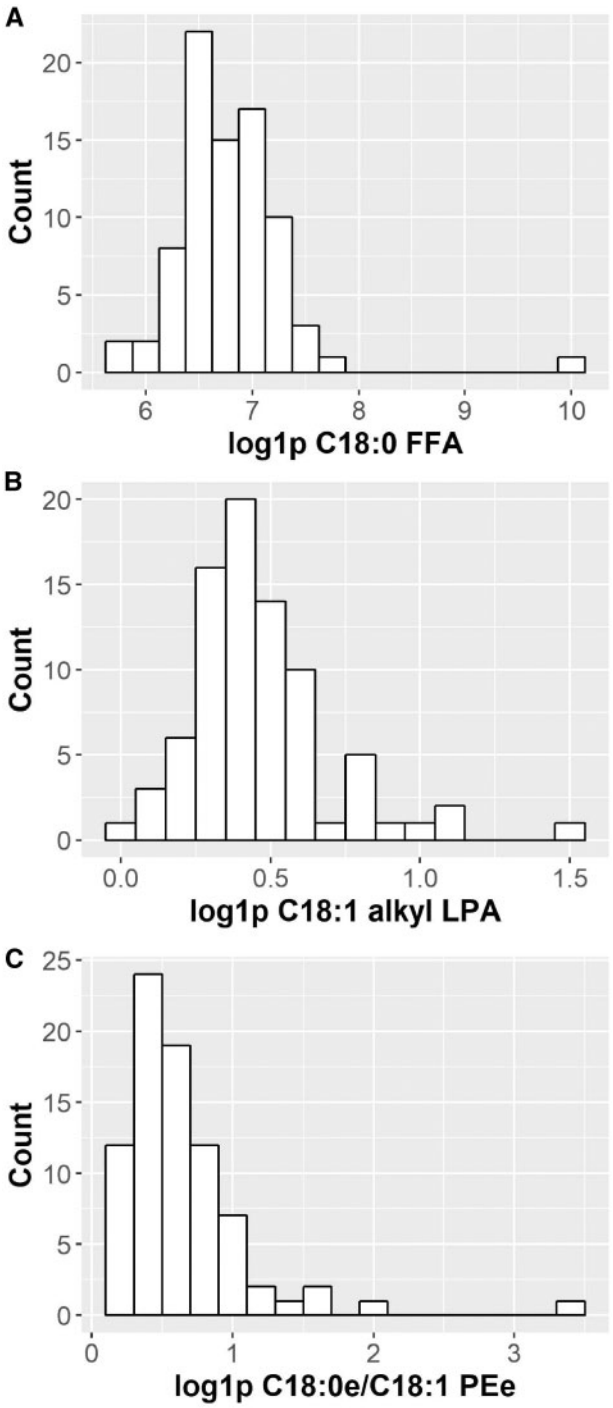

Figure 1: representative histograms of three lipid metabolite classes: (**A**) a fatty acid, C18:0 FFA; (**B**) a lysolipid, C18:1 alkyl LPA; and (**C**) a phospholipid, C18:0e/C18:1 PEe

of two phospholipid metabolites associated with methylation at six different CpG sites, shown by the data points above the red genome-wide significance threshold. Prior to adjustment of P-values for multiple hypotheses testing, this metabolite was associated with 10 338 CpG sites throughout the genome. Four of the false discovery rate (FDR) significant CpG sites associated with C18:0/C20:4 alkyl PA were also related to C18:1 LPI.

Several CpG sites had significant relationships with one phospholipid (C18:0/C20:4 alkyl PA) and/or two lysolipid (C16:0 alkyl LPA, C18:1 LPI) metabolites (lysolipids are phospholipid breakdown products). For instance, cg12106728 was negatively associated with C18:0/C20:4 alkyl PA ($\beta = -1.014$, FDR $P = 0.005$) and C18:1 LPI ($\beta = -1.667$, FDR $P = 0.003$). A CpG site that maps to *CTDSP2* and *MIR26A2*, cg17169243, was inversely related to C18:0/C20:4 alkyl PA ($\beta = -0.454$, FDR $P = 0.019$), C18:1 LPI ($\beta = -0.807$, FDR $P = 0.002$), and C16:0 alkyl LPA ($\beta = -0.366$, FDR $P = 0.021$). cg19220754, located in the body of *TNPO1*, was hypomethylated with increased C18:0/C20:4 alkyl PA ($\beta = -1.843$, FDR $P = 0.019$) and C18:1 LPI ($\beta = -2.962$, FDR $P = 0.019$). cg24175823 was negatively associated with C18:0/C20:4 alkyl PA ($\beta = -1.341$, FDR $P = 0.002$), C18:1 LPI ($\beta = -2.039$, FDR $P = 0.018$), and C16:0 alkyl LPA ($\beta = -1.024$, FDR $P = 0.018$). Out of the 28 total significant relationships between metabolites and CpG sites, 22 had negative regression coefficients, suggesting that increased lipidomic levels in maternal blood are generally associated with lower DNA methylation in their newborn children.

Although most of the significant associations were negative, some of the positive relationships observed between maternal metabolite levels and infant DNA methylation had the largest effect sizes. A CpG site that maps to *SEPT2* and *HDLBP*, cg16787284, was positively related to C16:0e/C18:1 PSe ($\beta = 25.723$, FDR $P = 0.026$) and C18:0e/C18:1 PEe ($\beta = 1.154$, FDR $P = 0.021$). cg22539279, located in *NEK11* and *ASTE1*, was also hypermethylated with increased C16:0e/C18:1 PSe ($\beta = 23.200$, FDR $P = 0.021$).

In addition, seven of the FDR-significant relationships remained after using the more stringent Bonferroni adjustment. These included the associations of cg10874881 and cg16597728 with the phospholipid C16:0/C20:4 PS, cg12106728 and cg24175823 with the phospholipid C18:0/C20:4 alkyl PA, cg21883754 with the phospholipid ether C16:0e/C18:1 PSe, and cg12106728 and cg17169243 with the lysolipid C18:1 LPI.

Pathway Analysis of Gene Hits

The maternal metabolites significantly associated with newborn methylation included complex lipids and products of lipid degradation, side products generated from choline and fatty acid release for the developing fetus (Fig. 4A and B). These lipids provide a methyl source for DNA methylation and drive growth, providing lipid building blocks and energy (Fig. 4B). To determine the biological relevance of the metabolomic and methylation findings, we ran pathway analysis of the genes for which the significant CpG hits were mapped (Table 4). The most common molecular functions relevant to the genes included binding and catalytic activity. In addition, the genes were involved in a range of biological processes at the cellular and metabolic levels and participated in biological regulation, response to stimulus, and localization. The genes *BDNF*, *PC*, and *SEPT2* have been related to a variety of pathways, including Huntington disease, pyruvate metabolism, and Parkinson disease, respectively.

Discussion

In this study, we characterized the lipidomic profiles of pregnant women from the CHAMACOS cohort, building upon previous research that identified associations of metabolomic data with phthalates, endocrine disrupting chemicals, and maternal pre-pregnancy BMI [18]. We focused on maternal metabolite levels assessed around 26 weeks gestation, rather than at child delivery, due to the availability of existing metabolomic data and more importantly, to better capture exposures relevant during fetal development that could relate to newborn epigenetic

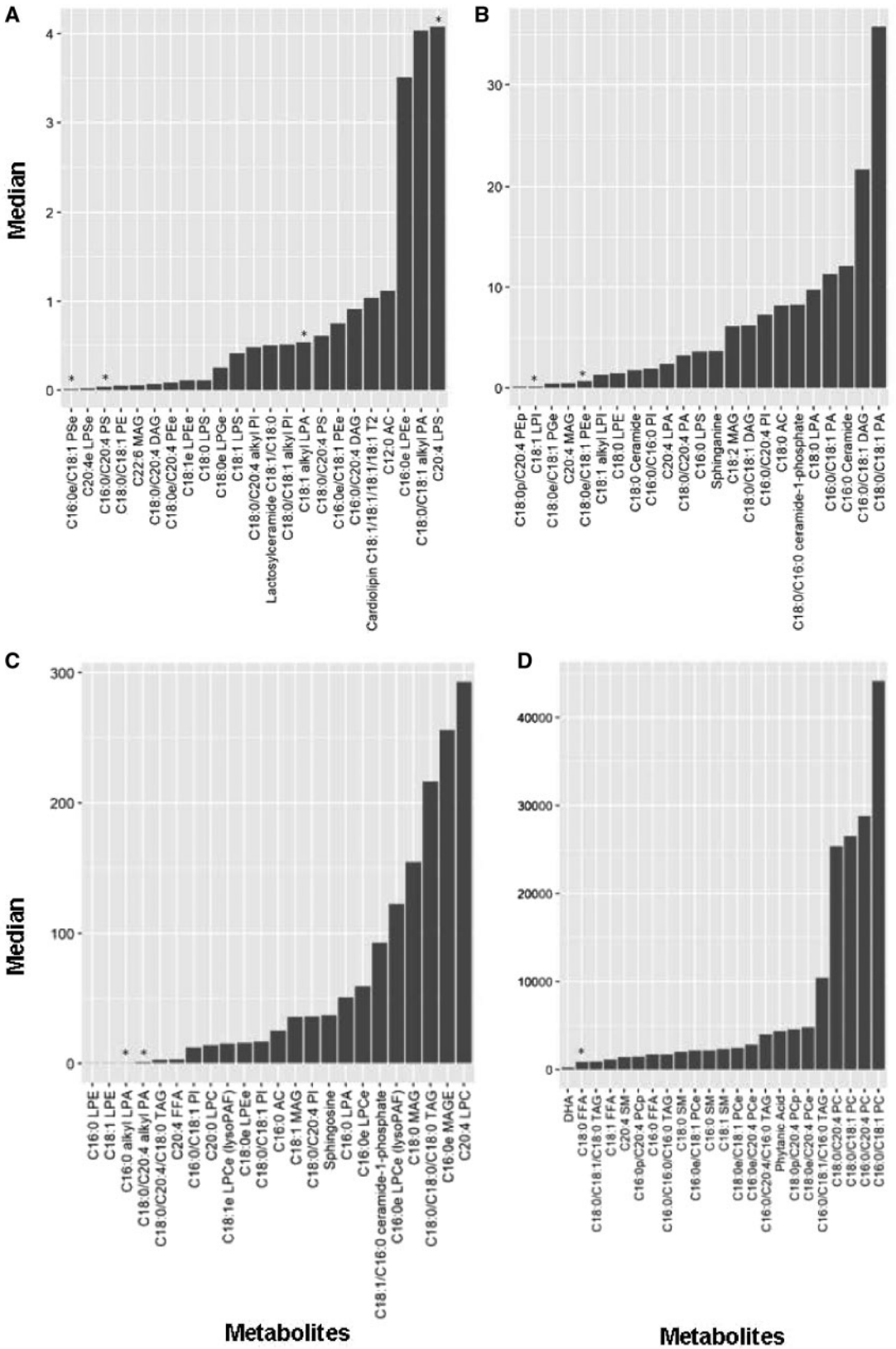

Figure 2: median levels of metabolites with ranges in the (**A**) first (lowest), (**B**) second, (**C**) third, and (**D**) fourth (highest) quartiles.
*Represents metabolites that were significantly associated with DNA methylation in newborns

Table 2: distribution of lipid metabolites in plasma of CHAMACOS mothers ($N = 81$)

Metabolite	Pathway	Median	IQR
C16:0/C18:1 DAG	Diacylglycerol	21.56	[14.44, 30.06]
C16:0/C20:4 DAG	Diacylglycerol	0.91	[0.57, 1.35]
C18:0/C18:1 DAG	Diacylglycerol	6.22	[3.83, 9.53]
C18:0/C20:4 DAG	Diacylglycerol	0.07	[0.04, 0.12]
C12:0 AC	Fatty acid	1.12	[0.94, 1.41]
C16:0 AC	Fatty acid	25.18	[14.61, 40.10]
C16:0 FFA	Fatty acid	1660.35	[1282.00, 2199.70]
C18:0 AC	Fatty acid	8.19	[4.08, 11.94]
C18:0 FFA	Fatty acid	817.78	[664.32, 1159.16]
C18:1 FFA	Fatty acid	1109.79	[732.70, 1765.65]
C20:4 FFA	Fatty acid	2.96	[2.04, 3.78]
DHA	Fatty acid	232.88	[39.63, 752.88]
Phytanic acid	Fatty acid	4337.56	[201.02, 10 241.48]
C16:0 alkyl LPA	Lysolipid	0.46	[0.20, 0.85]
C16:0 LPA	Lysolipid	50.51	[40.01, 59.68]
C16:0 LPE	Lysolipid	0.28	[0.15, 0.61]
C16:0 LPS	Lysolipid	3.61	[2.37, 4.75]
C16:0e LPCe	Lysolipid	59.15	[34.00, 85.07]
C16:0e LPCe (lysoPAF)	Lysolipid	122.48	[83.12, 171.97]
C16:0e LPEe	Lysolipid	3.51	[2.56, 4.35]
C18:0 LPA	Lysolipid	9.77	[7.42, 13.39]
C18:0 LPE	Lysolipid	1.47	[0.91, 2.80]
C18:0 LPS	Lysolipid	0.11	[0.07, 0.17]
C18:0e LPEe	Lysolipid	15.59	[2.80, 24.27]
C18:0e LPGe	Lysolipid	0.25	[0.02, 1.12]
C18:1 alkyl LPA	Lysolipid	0.54	[0.37, 0.73]
C18:1 alkyl LPI	Lysolipid	1.3	[0.62, 2.88]
C18:1 LPE	Lysolipid	0.43	[0.21, 1.08]
C18:1 LPI	Lysolipid	0.12	[0.08, 0.17]
C18:1 LPS	Lysolipid	0.41	[0.19, 0.57]
C18:1e LPCe (lysoPAF)	Lysolipid	15.01	[2.96, 24.26]
C18:1e LPEe	Lysolipid	0.11	[0.07, 0.20]
C20:0 LPC	Lysolipid	13.84	[6.64, 19.75]
C20:4 LPA	Lysolipid	2.35	[1.68, 2.91]
C20:4 LPC	Lysolipid	292.84	[196.19, 367.39]
C20:4 LPS	Lysolipid	4.08	[3.60, 4.64]
C20:4e LPSe	Lysolipid	0.02	[0.01, 0.05]
C16:0e MAGE	Monoacylglycerol	255.78	[206.49, 327.70]
C18:0 MAG	Monoacylglycerol	154.63	[72.42, 199.03]
C18:1 MAG	Monoacylglycerol	35.7	[21.99, 64.30]
C18:2 MAG	Monoacylglycerol	6.14	[1.27, 12.82]
C20:4 MAG	Monoacylglycerol	0.47	[0.24, 0.88]
C22:6 MAG	Monoacylglycerol	0.05	[0.03, 0.09]
C16:0/C16:0 PI	Phospholipid	1.9	[1.03, 3.60]
C16:0/C18:1 PA	Phospholipid	11.3	[10.35, 13.37]
C16:0/C18:1 PC	Phospholipid	44 132.92	[37 269.07, 52 132.60]
C16:0/C18:1 PI	Phospholipid	12.26	[7.23, 20.11]
C16:0/C20:4 PC	Phospholipid	28 780.3	[24 703.64, 34 127.57]
C16:0/C20:4 PI	Phospholipid	7.28	[4.30, 12.53]
C16:0/C20:4 PS	Phospholipid	0.04	[0.02, 0.07]
C16:0e/C18:1 PCe	Phospholipid	2154.21	[1889.12, 2520.64]
C16:0e/C18:1 PEe	Phospholipid	0.75	[0.30, 1.02]
C16:0e/C18:1 PSe	Phospholipid	0.01	[0.01, 0.02]
C16:0e/C20:4 PCe	Phospholipid	2833.01	[2487.41, 3331.83]
C16:0p/C20:4 PCp	Phospholipid	1441.64	[1198.07, 1707.04]
C18:0/C18:1 alkyl PA	Phospholipid	4.04	[3.38, 4.59]
C18:0/C18:1 alkyl PI	Phospholipid	0.51	[0.30, 0.88]
C18:0/C18:1 PA	Phospholipid	35.8	[32.05, 42.42]
C18:0/C18:1 PC	Phospholipid	26 515.68	[22 344.95, 30 784.37]
C18:0/C18:1 PE	Phospholipid	0.05	[0.03, 0.09]
C18:0/C18:1 PI	Phospholipid	16.47	[9.57, 28.92]
C18:0/C20:4 alkyl PA	Phospholipid	0.82	[0.68, 1.05]

(continued)

Table 2: (continued)

Metabolite	Pathway	Median	IQR
C18:0/C20:4 alkyl PI	Phospholipid	0.48	[0.30, 0.89]
C18:0/C20:4 PA	Phospholipid	3.23	[2.75, 3.96]
C18:0/C20:4 PC	Phospholipid	25 368.63	[22 167.33, 28 643.58]
C18:0/C20:4 PI	Phospholipid	36.05	[21.71, 52.25]
C18:0/C20:4 PS	Phospholipid	0.61	[0.18, 0.91]
C18:0e/C18:1 PCe	Phospholipid	2440.81	[1981.18, 2796.77]
C18:0e/C18:1 PEe	Phospholipid	0.7	[0.44, 1.18]
C18:0e/C18:1 PGe	Phospholipid	0.45	[0.03, 2.34]
C18:0e/C20:4 PCe	Phospholipid	4815.13	[4010.52, 5666.14]
C18:0e/C20:4 PEe	Phospholipid	0.09	[0.03, 0.16]
C18:0p/C20:4 PCp	Phospholipid	4569.48	[3835.01, 5374.80]
C18:0p/C20:4 PEp	Phospholipid	0.1	[0.04, 0.16]
Cardiolipin C18:1/18:1/18:1/18:1 T2	Phospholipid	1.04	[0.80, 1.31]
C16:0 ceramide	Sphingolipid	12.11	[9.50, 13.92]
C16:0 SM	Sphingolipid	2178.44	[1830.64, 2544.65]
C18:0 ceramide	Sphingolipid	1.79	[1.37, 2.36]
C18:0 SM	Sphingolipid	1997.51	[1135.78, 2943.23]
C18:0/C16:0 ceramide-1-phosphate	Sphingolipid	8.26	[7.32, 10.12]
C18:1 SM	Sphingolipid	2325.2	[2009.34, 2810.72]
C18:1/C16:0 ceramide-1-phosphate	Sphingolipid	92.51	[78.65, 109.94]
C20:4 SM	Sphingolipid	1400.77	[881.66, 1765.40]
Lactosylceramide C18:1/C18:0	Sphingolipid	0.5	[0.28, 0.77]
Sphinganine	Sphingolipid	3.64	[1.38, 11.14]
Sphingosine	Sphingolipid	36.95	[19.04, 51.62]
C16:0/C16:0/C16:0 TAG	Triacylglycerol	1690.96	[958.05, 2779.60]
C16:0/C18:1/C16:0 TAG	Triacylglycerol	10 433.19	[7397.58, 13 658.95]
C16:0/C20:4/C16:0 TAG	Triacylglycerol	3969.7	[2889.16, 5224.91]
C18:0/C18:0/C18:0 TAG	Triacylglycerol	216.26	[121.32, 356.69]
C18:0/C18:1/C18:0 TAG	Triacylglycerol	868.13	[430.36, 1782.46]
C18:0/C20:4/C18:0 TAG	Triacylglycerol	2.72	[1.61, 9.12]

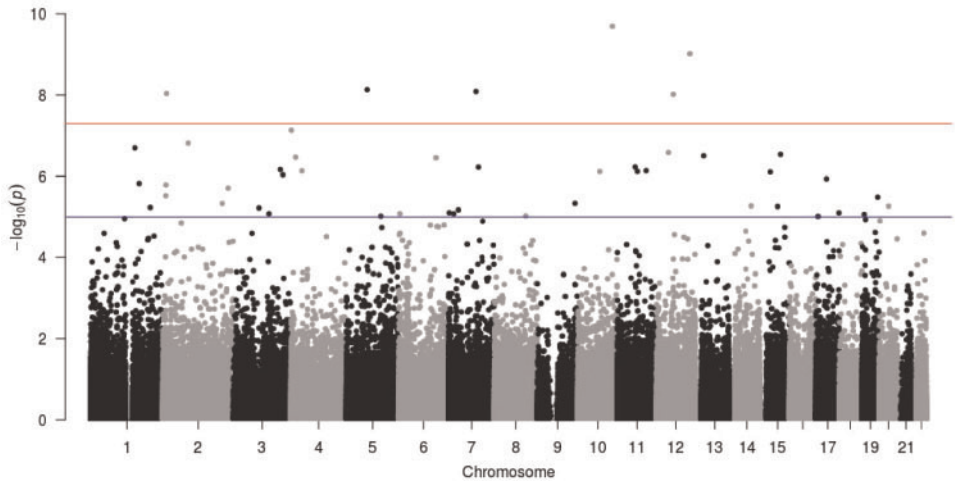

Figure 3: manhattan plot of CpG sites of CHAMACOS newborns associated with C18:0/C20:4 alkyl PA levels in the blood of their mothers during pregnancy. Red line represents genome-wide significance threshold of $-\log_{10}$(5.00E-08), while the blue line corresponds to the suggestive threshold of $-\log_{10}$(1.00E-05)

profiles. The 92 lipid metabolites assessed had broad ranges of levels in the women's plasma. We further examined the relationship between maternal metabolite counts and offspring DNA methylation. Out of the lipid metabolites assessed, we identified significant and predominantly negative associations among four phospholipids, four lysolipids, and a fatty acid with newborn methylation. In addition, several of the significant metabolites were related to multiple CpG sites. For example, the phospholipids C16:0/C20:4 PS and C18:0/C20:4 alkyl PA were both associated with 6 CpG sites. These results strengthen the evidence that maternal metabolites, particularly lysolipids and phospholipids, are related to reduced offspring DNA methylation at CpG sites in genes involved in a variety of biological processes, including catalytic and binding activities.

The wide range in lipid metabolite levels observed in CHAMACOS is similar to a study conducted in 40 mother–child dyads from a clinical birth cohort in Michigan [5]. Marchlewicz *et al.* examined acylcarnitine, free fatty acid, and amino acid

Table 3: significant results of the relationship between prenatal maternal metabolite levels in plasma and newborn cord blood DNA methylation

CpG sites	Chromosome	*N*	*β*	SE	FDR *P*-value	Significant exposures	Genes	Gene location	Relation to CpG island
cg02496111	16	81	−3.172	0.467	0.012	C16:0/C20:4 PS			Island
cg03168497	17	81	−2.118	0.330	0.021	C18:1 alkyl LPA	*MYCBPAP*	Body	Island
cg04108939	1	80	1.169	0.187	0.039	C20:4 LPS	*BEST4*	Body	Island
cg06494167	2	80	−1.428	0.214	0.018	C18:1 LPI	*RMND5A*	TSS1500	Island
cg07370087	1	81	0.469	0.076	0.044	C18:0 FFA			
cg08838610	17	81	−3.662	0.589	0.040	C16:0/C20:4 PS	*RPAIN*, *NUP88*	TSS200, 1st exon	Island
cg09931872	10	80	−2.409	0.365	0.018	C16:0/C20:4 PS	*WDFY4*	5′UTR	
cg10528455	2	81	0.492	0.076	0.019	C18:0/C20:4 alkyl PA			
cg10874881	7	80	−2.546	0.346	**0.002**	C16:0/C20:4 PS	*POU6F2*	Body	
cg12106728	12	79	−1.014	0.144	**0.005**	C18:0/C20:4 alkyl PA			Island
		79	−1.667	0.229	**0.003**	C18:1 LPI			
cg14630692	9	81	−0.305	0.047	0.019	C16:0 alkyl LPA	*URM1*	Body	
cg14897096	11	78	−1.911	0.286	0.018	C16:0/C20:4 PS	*PC*	5′UTR	N_Shelf
cg15710245	11	79	−0.731	0.114	0.024	C18:0e/C18:1 PEe	*BDNF*	TSS200, TSS1500, body, 5′UTR	Island
cg16597728	20	79	−2.063	0.293	**0.005**	C16:0/C20:4 PS	*APCDD1L*	Body	S_Shore
cg16787284	2	81	25.723	4.056	0.026	C16:0e/C18:1 PSe	*SEPT2*, *HDLBP*	TSS200, 5′UTR	Island
		81	1.154	0.180	0.021	C18:0e/C18:1 PEe			
cg17169243	12	79	−0.366	0.057	0.021	C16:0 alkyl LPA	*CTDSP2*, *MIR26A2*	Body, TSS1500	
		79	−0.454	0.070	0.019	C18:0/C20:4 alkyl PA			
		79	−0.807	0.105	**0.002**	C18:1 LPI			
cg19220754	5	81	−1.843	0.281	0.019	C18:0/C20:4 alkyl PA	*TNPO1*	Body	Island
		81	−2.962	0.456	0.019	C18:1 LPI			
cg21883754	19	79	−21.976	2.924	**0.002**	C16:0e/C18:1 PSe	*TTYH1*	TSS200	N_Shore
cg22539279	3	81	23.200	3.607	0.021	C16:0e/C18:1 PSe	*NEK11*, *ASTE1*	TSS200	Island
cg24175823	10	77	−1.024	0.154	0.018	C16:0 alkyl LPA			Island
		77	−1.341	0.180	**0.002**	C18:0/C20:4 alkyl PA			
		77	−2.039	0.306	0.018	C18:1 LPI			
cg27492749	7	76	−1.548	0.235	0.019	C18:0/C20:4 alkyl PA	*SGCE*, *PEG10*	Body, TSS1500	Island

Bolded FDR *P*-values indicate CpGs that were also significant based on the more conservative Bonferroni correction (uncorrected *P*-value $<1.36 \times 10^{-9}$).
β represents regression coefficients of the relationship between log(1+*x*) transformed maternal prenatal metabolites and newborn DNA methylation *M*-values, adjusting for child sex, batch, and white blood cell composition.

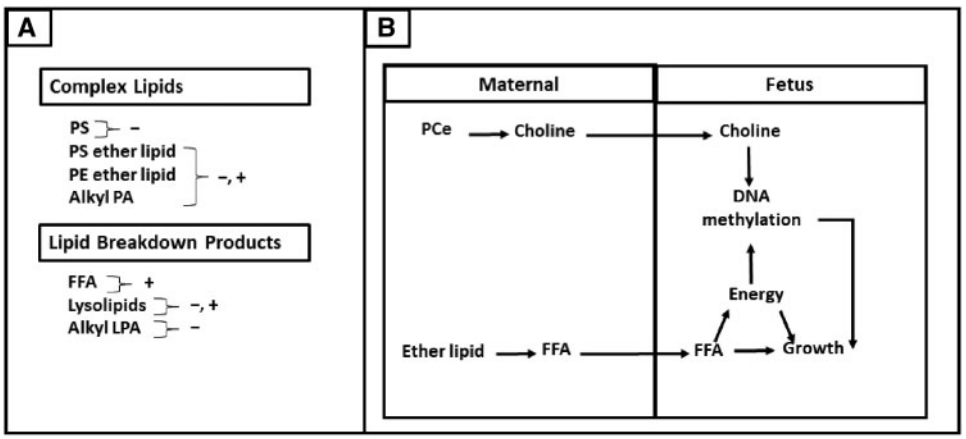

Figure 4: diagram of the significant positive (+) and negative (−) associations observed between maternal metabolites during pregnancy and newborn DNA methylation (**A**) and the biological pathways whereby maternal lipid levels could impact DNA methylation of their children (**B**)

metabolite levels of mothers during the first trimester and at delivery, as well as of their children at birth. They observed wide distributions of free fatty acid levels similar to CHAMACOS, with medians ranging from 0.00 to 152.70 nmol/ml and 0.00 to 247.90 nmol/ml for the maternal first trimester and delivery metabolites, respectively. In CHAMACOS mothers, median free fatty acid metabolite counts ranged from 1.12 to 4337.56. Higher levels in the CHAMACOS cohort could be attributed to differences in study populations; since CHAMACOS participants have a high prevalence of obesity and are Mexican-

Table 4: pathway information for genes with CpG sites significantly associated with maternal metabolite levels

Gene	Molecular function	Biological process	Cellular component	Protein class	Pathway
BDNF	Binding; molecular transducer activity	Biological regulation; cellular component organization or biogenesis; cellular process; multicellular organismal process; response to stimulus; signaling	Cell; extracellular region; membrane; organelle; synapse	Signaling molecule	Huntington disease; metabotropic glutamate receptor group II pathway
BEST4				Transporter	
CTDSP2	Catalytic activity				
NEK11		Cellular process	Cell; organelle	Transferase	
NUP88		Localization	Cell; organelle; protein-containing complex	Transporter	
PC	Catalytic activity	Metabolic process	Cell	Ligase	Pyruvate metabolism
RMND5A	Catalytic activity	Metabolic process	Cell; organelle; protein-containing complex		
SEPT2	Binding; catalytic activity	Cellular component organization or biogenesis; cellular process; localization	Cell; membrane; protein-containing complex	Cytoskeletal protein; enzyme modulator	Parkinson disease
SGCE	Binding	Developmental process		Calcium-binding protein	
TNPO1	Binding; transporter activity	Localization	Cell; membrane; organelle	Transfer/carrier protein; transporter	
TTYH1	Transporter activity		Cell	Transporter	

APCDD1L, *MYCBPAP*, *POU6F2*, *URM1*, and *WDFY4* did not map to any pathway data in PANTHER.

American as compared to the population in the Marchlewicz *et al.* study, which was mostly Caucasian; and the species of free fatty acid metabolites assessed in each cohort.

In addition to determining the distributions of lipid metabolites in maternal plasma, another critical objective of this study was to assess the relationship between maternal blood lipid levels and offspring DNA methylation at birth. Using a targeted approach, previous research in animal models has demonstrated associations between maternal fatty acid diet and increased DNA methylation of the fatty acid desaturase 2 (*Fads2*) gene in the liver of offspring, which encodes for Δ6 desaturase, a rate limiting enzyme in polyunsaturated fatty acid synthesis [16, 17]. A similar study in humans of the effect of fatty acid supplementation showed no significant differences when comparing cord blood promoter methylation profiles of offspring of pregnant women receiving DHA or those in the placebo group [20]. In addition, sphingosine-1-phosphate, a sphingolipid, has been linked at the cellular level to another epigenetic mechanism, histone acetylation [21].

Only a couple of studies have examined the association between blood lipid metabolite profiles and DNA methylation in humans. Petersen *et al.* [6] assessed concurrent metabolomics profiles, including lipid metabolites, and DNA methylation levels in 1814 adults in Germany. Using methylation data measured by the Infinium HumanMethylation 450 BeadChip platform, as used in this study, and utilizing genome-wide single nucleotide polymorphism (SNP) data, they were able to identify a significant and positive relationship, which also showed a strong genetic effect, between the phospholipid PC aa C38:4 and cg11250194. In addition, several CpG site and metabolite associations were discovered after correcting for genetic effects, including a positive relationship between cg17901584 and the phospholipid PC ae C36:5. We also observed significant associations with phospholipid metabolite levels in the CHAMACOS cohort. Specifically, maternal levels of C16:0/C20:4 PS, C16:0e/C18:1 PSe, C18:0e/C18:1 PEe, and C18:0/C20:4 alkyl PA were all related to newborn DNA methylation.

In CHAMACOS, we observed predominantly negative relationships between maternal second trimester metabolite counts and newborn DNA methylation. Marchlewicz *et al.* [5] conducted a similar study in 40 predominantly white (85%) mothers and their children from Michigan. They examined whether maternal metabolomic profiles are associated with cord blood methylation globally, measured by *LINE-1* and *LUMA*, and at genes relevant for growth, including the imprinted genes *H19* and *IGF2*. They observed that maternal fatty acids measured during the first trimester were significantly and positively correlated with infant methylation at *LINE-1*, *IGF2*, *ESR1*, and *PPARα*, and negatively correlated with *H19* and *LUMA*. Additional correlations were found for maternal delivery and cord blood metabolite levels with newborn methylation, with similar directions of association. Although we were not able to replicate the results observed in the Marchelwizc *et al.* study since they used a candidate gene approach, we were also able to observe a significant relationship between maternal lipid levels and imprinted gene methylation in newborns. Specifically, the relationship between cg27492749, a CpG site within another imprinted gene (*SGCE*), and the lipid metabolite C18:0/C20:4 alkyl PA. Taken together, these studies provide preliminary evidence of associations between maternal metabolomic environments during pregnancy and DNA methylation status of their offspring.

Several of the genes with significant CpG sites related to maternal metabolite levels in the CHAMACOS cohort are involved

in biological pathways associated with early life development. For instance, the *BDNF* gene encodes for the growth factor, brain-derived neurotrophic factor, which is important in the development of the central and peripheral nervous systems of infants [22]. Human septins, including the protein encoded by *SEPT2*, can assemble into higher-order cytoskeletal structures, such as filaments and bundles, which are necessary to the growing fetus [23]. In addition, the *PC* gene encodes for the enzyme pyruvate carboxylase, which fuels the tricarboxylic acid cycle, the major pathway that drives the biosynthesis of amino acids [24].

The statistically significant lipids in this study can be linked with DNA methylation in several ways. First, the process of lipid degradation releases fatty acids and lysolipids, as well as choline, which is transported to the developing infant. Choline serves as a major methyl source for DNA methylation and is also necessary for the synthesis of new biological membranes for rapidly dividing cells. The observed associations between DNA methylation and lipid degradation products may be possible because the metabolic flux is relatively slow or their abundance is low. In contrast, we did not find associations for the phosphatidylcholine (PC) or triacylglycerol (TG) metabolites, which serve as major sources of either fatty acids or choline [25, 26]. The metabolic flux through PC and TG metabolism and their relative abundance is relatively high (Fig. 2), which could obscure the association. Second, fatty acids provide a major energy source which drives growth in the developing fetus. Third, ether lipids regulate cell differentiation and cellular signaling [27]. Ether lipids are an integral component of lipid membranes, provide enhanced integrity at lipid raft microdomains, and are important for membrane fusion and trafficking.

A limitation of this study includes the relatively small sample size, which could limit the power to detect significant differences based on the large number of tests performed. However, our study has more power than the most comparable study [5], which has half the number of mother–child dyads with metabolomic and DNA methylation data. Another limitation is the absence of data on child metabolomic profiles at birth. However, given that maternal lipid metabolites can be transferred to the developing fetus via the placenta and that the prenatal period is a time of epigenetic remodeling, we feel that exploring the relationship between maternal metabolite levels during pregnancy, a sensitive time for epigenetic changes, is more relevant. In addition, although DNA methylation is the most commonly studied epigenetic mechanism, maternal lipid metabolites could also be related to other epigenetic marks in children, including histone modifications.

The main strengths of this study include the use of multiple 'omics' data from the well-characterized CHAMACOS cohort, including the assessment of numerous classes of lipids, which expanded upon the fatty acids that have been previously assessed in relation to infant methylation data. This allowed for the discovery of novel associations between maternal metabolite levels, including phospholipids and lysolipids, and newborn DNA methylation, which could have implications on child health. Previous studies [28, 29] in pregnant women with poor birth outcomes compared to controls revealed differences in metabolomic profiles of lipid metabolites. In addition, lower levels of lysophospholipids, phospholipids, and monoacylglycerols during early pregnancy have been associated with macrosomia [30]. Epigenetics could be one mechanism whereby *in utero* exposure to maternal metabolites could impact health in early life. Future research should further explore the potential role of maternal metabolomic profiles on offspring DNA methylation and its role in child health.

Methods

Study Population

Study participants included women and children from the CHAMACOS study, a longitudinal birth cohort originally aimed at examining the relationship of pesticide and environmental chemical exposure with the health and development of Mexican-American children from Salinas Valley, CA. A more in depth discussion of the study population has been characterized previously in Eskenazi et al. [19]. From 1999 to 2000, a total of 601 pregnant women were enrolled, resulting in the delivery of 527 liveborn singletons. Two pregnancy interviews were conducted at an average of 13.4 and 26.0 weeks gestation, with an additional visit after delivery. This study includes the 81 mother–child pairs with both metabolomic data from blood collected at the second pregnancy visit and with newborn Illumina HumanMethylation 450K BeadChip data. The University of California, Berkeley Committee for Protection of Human Subjects approved all study protocols and written informed consent was obtained from the CHAMACOS mothers.

Pregnancy Metabolite Measurements

Biological samples collected from 115 CHAMACOS women around 26 weeks gestation (mean = 26.4, SD = 3.2) were analysed to characterize maternal metabolomics profiles during pregnancy. Detailed descriptions of the metabolomics analyses in the CHAMACOS cohort have been previously described in Zhou et al. [18]. Briefly, blood from the women was analysed in duplicates by selected reaction monitoring (SRM) liquid chromatography and triple quadrupole mass spectrometry, a method previously validated by the Nomura research group [31]. SRM was used to identify metabolites, which were measured using the area under the curve. C_{12} MAGE, Pentadecanoic acid, and D^3N^{15} Serine were utilized in the normalization of plasma nonpolar positive, plasma nonpolar negative, and plasma polar metabolites, respectively. Laboratory and field blanks, as well as internal standards, were included to ensure quality, and repeat samples had good reproducibility (coefficients of variation $\leq$ 3–15%). For this study, we focused on the plasma metabolites involved in lipid pathways since lipids are involved in diverse biological activities including metabolic and structural functions, inflammation, signaling, and endocrine regulation [32].

Cord Blood Collection and Processing

At delivery, hospital staff collected cord blood in two types of vacutainers, one coated in heparin (green top) and the other without the anticoagulant (red top). Blood clots were aliquoted from the nonheparinized vacutainers and were stored at −80°C until use in DNA isolation.

DNA Preparation

DNA isolation of anticoagulant-free umbilical cord blood clot samples was performed using QIAamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) following the manufacturer's protocol, with the exception of small modifications that were previously described in Holland et al. [33].

450K BeadChip DNA Methylation Analysis

DNA samples of 1 μg were bisulfite converted using Zymo Bisulfite Conversion Kits (Zymo Research, Irvine, CA), whole

genome amplified, enzymatically fragmented, purified, and applied to Illumina Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA) following the manufacturer's instructions [34]. Samples were randomized across assay wells, chips, and plates to prevent batch bias. Robotics handled the 450K BeadChips, which were analysed using the Illumina Hi-Scan system. DNA methylation was assessed at 485 512 CpG sites.

Probe signal intensities were extracted by Illumina GenomeStudio software (version XXV2011.1, Methylation Module 1.9) methylation module and background subtracted. A variety of quality assurance and control measures were implemented and have been previously described in Yousefi et al. [35], such as determination of assay repeatability and batch effects. The All Sample Mean Normalization algorithm [35], adjusted for color channel bias, batch effects and differences in Infinium chemistry. In addition, Beta Mixture Quantile normalization was used to account for differences between the two Illumina probes [36]. Samples were retained if 95% of sites assayed had detection *P*-values greater than 0.01. Criteria for removal included: (i) sites with annotated probe SNPs ($n = 65$) and with common SNPs (minor allele frequency >5%) within 50 bp of the target identified in the MXL (Mexican ancestry in Los Angeles, CA) HapMap population ($n = 49\,748$); (ii) sites mapped to the X ($n = 10\,708$) and Y ($n = 95$) chromosomes [14]; (iii) cross-reactive probes identified by Chen *et al.* [37] ($n = 26\,950$); and (iv) probes where 95% of samples had detection *P*-values greater than 0.01 ($n = 460$). A total of 398 483 CpG sites remained for analysis. Methylation beta values across sites were logit transformed to the *M*-value scale to more accurately adhere to modeling assumptions [38]. Methylation observations greater or less than three times the interquartile range for a given CpG site were removed prior to regression analyses to reduce the influence of methylation outliers.

Statistical Analysis

To examine the distributions of individual metabolites in maternal blood during pregnancy, we computed descriptive statistics. We used Student's *t*-tests to compare methylation profiles between CHAMACOS newborns with methylation data that were included ($N = 81$) and excluded ($N = 288$) in the current analyses, with results indicating no significant differences in methylation between the two groups. There was no difference between the subset of the participants included in this study and the rest of the CHAMACOS cohort.

To determine the relationship between maternal pregnancy metabolomics profiles as the exposure and 450K DNA methylation of their newborn children as the outcome, we fit *limma* linear models with empirical Bayes variance shrinkage [39]. To reduce the influence of metabolite outliers, all 92 maternal lipid metabolites were log(1+*x*) transformed. We adjusted for sex and batch in each of the models. Since DNA methylation has been observed to vary by cell type [40], we also accounted for white blood cell type proportions in statistical models. To estimate cord blood proportions of seven white blood cell types, including nucleated red blood cells, we utilized a cord blood reference dataset from Johns Hopkins [41]. We adjusted *P*-values for multiple hypotheses testing using the Benjamini–Hochberg FDR threshold for significance of 0.05 [42]. We also identified CpGs that were significant based on the more conservative Bonferroni correction (uncorrected *P*-value $<1.36 \times 10^{-9}$ to account for 36 660 436 tests). This analysis was performed using R statistical computing software (v3.5.1) [43].

Gene Ontology Analysis

We used the online tool PANTHER (protein annotation through evolutionary relationship) [44] to classify the function and relevant pathways of genes with FDR significant CpG sites related to lipid metabolites. Briefly, the list of genes was entered into the 'Gene List Analysis' tool in PANTHER and the functional classification for each gene was generated. Gene ontology related to molecular function, biological process, cellular component, protein class, and pathway was available for most of the genes identified in the regression results.

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References

1. Wild CP. The exposome: from concept to utility. *Int J Epidemiol* 2012;**41**:24–32.
2. Zierer J, Menni C, Kastenmüller G, Spector TD. Integration of 'omics' data in aging research: from biomarkers to systems biology. *Aging Cell* 2015;**14**:933–44.
3. Kretowski A, Ruperez FJ, Ciborowski M. Genomics and metabolomics in obesity and type 2 diabetes. *J Diabetes Res* 2016; **2016**:9415645.
4. Shin S, Fauman EB, Petersen A, Krumsiek J, Santos R, Huang J, Arnold M, Erte I, Forgetta V, Yang T, et al. An atlas of genetic influences on human blood metabolites. *Nat Genet* 2014;**46**: 543–50.
5. Marchlewicz EH, Dolinoy DC, Tang L, Milewski S, Jones TR, Goodrich JM, Soni T, Domino SE, Song PXK, Burant CF, et al. Lipid metabolism is associated with developmental epigenetic programming. *Sci Rep* 2016;**6**:34857.
6. Petersen A, Zeilinger S, Kastenmüller G, Römisch-Margl W, Brugger M, Peters A, Meisinger C, Strauch K, Hengstenberg C, Pagel P, et al. Epigenetics meets metabolomics: an epigenome-wide association study with blood serum metabolic traits. *Hum Mol Genet* 2014;**23**:534–45.
7. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, Bandyopadhyay S, Jones KN, Kelly S, Shaner RL, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. *J Lipid Res* 2010;**51**:3299–305.
8. Foley DL, Craig JM, Morley R, Olsson CA, Olsson CJ, Dwyer T, Smith K, Saffery R. Prospects for epigenetic epidemiology. *Am J Epidemiol* 2009;**169**:389–400.

9. Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005;**6**:597–610.
10. Murphy SK, Bassil CF, Huang Z. Main principles and outcomes of DNA methylation analysis. *Methods Mol Biol* 2013; **1049**:67–74.
11. Huen K, Calafat AM, Bradman A, Yousefi P, Eskenazi B, Holland N. Maternal phthalate exposure during pregnancy is associated with DNA methylation of LINE-1 and Alu repetitive elements in Mexican-American children. *Environ Res* 2016;**148**:55–62.
12. Solomon O, Yousefi P, Huen K, Gunier RB, Escudero-Fung M, Barcellos LF, Eskenazi B, Holland N. Prenatal phthalate exposure and altered patterns of DNA methylation in cord blood. *Environ Mol Mutagen* 2017;**58**:398–410.
13. Tindula G, Murphy SK, Grenier C, Huang Z, Huen K, Escudero-Fung M, Bradman A, Eskenazi B, Hoyo C, Holland N. DNA methylation of imprinted genes in Mexican-American newborn children with prenatal phthalate exposure. *Epigenomics* 2018;**10**:1011–26.
14. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richmond RC, Xu C-J, et al. DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet* 2016;**98**:680–96.
15. Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. *Reprod Toxicol* 2011;**31**:363–73.
16. Niculescu MD, Lupu DS, Craciunescu CN. Perinatal manipulation of α-linolenic acid intake induces epigenetic changes in maternal and offspring livers. *FASEB J* 2013;**27**:350–8.
17. Hoile SP, Irvine NA, Kelsall CJ, Sibbons C, Feunteun A, Collister A, Torrens C, Calder PC, Hanson MA, Lillycrop KA, et al. Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver. *J Nutr Biochem* 2013;**24**:1213–20.
18. Zhou M, Ford B, Lee D, Tindula G, Huen K, Tran V, Bradman A, Gunier R, Eskenazi B, Nomura DK, et al. Metabolomic markers of phthalate exposure in plasma and urine of pregnant women. *Front Public Health* 2018;**6**:298.
19. Eskenazi B, Bradman A, Gladstone EA, Jaramillo S, Birch K, Holland N. CHAMACOS, a longitudinal birth cohort study: lessons from the fields. *J Child Health* 2003;**1**:3–27.
20. Lee H, Barraza-Villarreal A, Hernandez-Vargas H, Sly PD, Biessy C, Ramakrishnan U, Romieu I, Herceg Z. Modulation of DNA methylation states and infant immune system by dietary supplementation with ω-3 PUFA during pregnancy in an intervention study. *Am J Clin Nutr* 2013;**98**:480–7.
21. Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S, et al. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* 2009;**325**:1254–7.
22. Rosas-Vargas H, Martínez-Ezquerro JD, Bienvenu T. Brain-derived neurotrophic factor, food intake regulation, and obesity. *Arch Med Res* 2011;**42**:482–94.
23. Mostowy S, Cossart P. Septins: the fourth component of the cytoskeleton. *Nat Rev Mol Cell Biol* 2012;**13**:183–94.
24. Lao-On U, Attwood PV, Jitrapakdee S. Roles of pyruvate carboxylase in human diseases: from diabetes to cancers and infection. *J Mol Med* 2018;**96**:237–47.
25. Coleman RA, Lee DP. Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* 2004;**43**:134–76.
26. Fagone P, Jackowski S. Phosphatidylcholine and the CDP-choline cycle. *Biochim Biophys Acta* 2013;**1831**:523.
27. Dean JM, Lodhi IJ. Structural and functional roles of ether lipids. *Protein Cell* 2018;**9**:196–206.
28. Heazell AEP, Bernatavicius G, Warrander L, Brown MC, Dunn WB. A metabolomic approach identifies differences in maternal serum in third trimester pregnancies that end in poor perinatal outcome. *Reprod Sci* 2012;**19**:863–75.
29. Lizewska B, Teul J, Kuc P, Lemancewicz A, Charkiewicz K, Goscik J, Kacerovsky M, Menon R, Miltyk W, Laudanski P. Maternal plasma metabolomic profiles in spontaneous preterm birth: preliminary results. *Mediat Inflamm* 2018;**2018**:9362820.
30. Ciborowski M, Zbucka-Kretowska M, Bomba-Opon D, Wielgos M, Brawura-Biskupski-Samaha R, Pierzynski P, Szmitkowski M, Wolczynski S, Lipinska D, Citko A, et al. Potential first trimester metabolomic biomarkers of abnormal birth weight in healthy pregnancies. *Prenat Diagn* 2014;**34**:870–7.
31. Patti GJ, Yanes O, Siuzdak G. Innovation: metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol* 2012;**13**:263–9.
32. Ibanez C, Mouhid L, Reglero G, Ramirez de Molina A. Lipidomics insights in health and nutritional intervention studies. *J Agric Food Chem* 2017;**65**:7827–42.
33. Holland N, Furlong C, Bastaki M, Richter R, Bradman A, Huen K, Beckman K, Eskenazi B. Paraoxonase polymorphisms, haplotypes, and enzyme activity in Latino mothers and newborns. *Environ Health Perspect* 2006;**114**:985–91.
34. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP, Gunderson KL, et al. High density DNA methylation array with single CpG site resolution. *Genomics* 2011;**98**:288–95.
35. Yousefi P, Huen K, Aguilar Schall R, Decker A, Elboudwarej E, Quach H, Barcellos L, Holland N. Considerations for normalization of DNA methylation data by Illumina 450K BeadChip assay in population studies. *Epigenetics* 2013;**8**:1141–52.
36. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, Beck S. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450K DNA methylation data. *Bioinformatics* 2013;**29**: 189–96.
37. Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, Gallinger S, Hudson TJ, Weksberg R. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation 450 microarray. *Epigenetics* 2013;**8**:203–9.
38. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, Lin SM. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinform* 2010;**11**:587.
39. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004;**3**:1–25.
40. Yousefi P, Huen K, Quach H, Motwani G, Hubbard A, Eskenazi B, Holland N. Estimation of blood cellular heterogeneity in newborns and children for epigenome-wide association studies. *Environ Mol Mutagen* 2015;**56**:751–8.
41. Bakulski KM, Feinberg JI, Andrews SV, Yang J, Brown S, L McKenney S, Witter F, Walston J, Feinberg AP, Fallin MD. DNA methylation of cord blood cell types: applications for mixed cell birth studies. *Epigenetics* 2016;**11**:354–62.
42. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach. *J R Stat Soc Ser B Stat Methodol* 1995;**57**:289–300.
43. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2018. http://www.R-project.org/.
44. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc* 2013;**8**:1551–66.